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(54) Title: METHODS TO IDENTIFY GROWTH DIFFERENTIATION FACTOR (GDF) RECEPTORS

(57) Abstract

The present invention provides receptors for the growth differentiation factor (GDF) family of growth factors and methods of idenfifying such receptors. Also included are methods of identifying antibodies which bind to the receptors, peptide fragments of the receptor which inhibit GDF binding, GDF receptor–binding agents capable of blocking GDF binding to the receptor. The receptors of the invention allow the identification of antagonists or agonists useful for agricultural and human therapeutic purposes.

METHODS TO IDENTIFY GROWTH DIFFERENTIATION FACTOR (GDF) RECEPTORS

1. *Field of the Invention*

This invention relates generally to ligand-receptor interactions and more specifically to growth differentiation factor receptor proteins and the ligands that bind to such receptors and methods of use therefor.

2. *Description of Related Art*

The transforming growth factor β (TGF-β) superfamily encompasses a group of structurally-related proteins which affect a wide range of differentiation processes during embryonic development. The family includes, Mullerian inhibiting substance (MIS), which is required for normal male sex development (Behringer, *et al., Nature*, 345:167, 1990), Drosophila decapentaplegic (DPP) gene product, which is required for dorsal-ventral axis formation and morphogenesis of the imaginal disks (Padgett, *et al., Nature*, 325:81 -84, 1987), the Xenopus Vg-1 gene product, which localizes to the vegetal pole of eggs ((Weeks, *et al., Cell*, 51:861-867, 1987), the activins (Mason, *et al., Biochem, Biophys. Res. Commun.*, 135:957-964, 1986), which can induce the formation of mesoderm and anterior structures in Xenopus embryos (Thomsen, *et al., Cell*, 63:485, 1990), and the bone morphogenetic proteins (BMPs, osteogenin, OP-1) which can induce de novo cartilage and bone formation (Sampath, *et al., J. Biol. Chem.*, 265:13198, 1990). The TGF-βs can influence a variety of differentiation processes, including adipogenesis, myogenesis, chondrogenesis, hematopolesis, and epithelial cell differentiation (for review, see Massague, *Cell* 49:437, 1987).

The proteins of the TGF-β family are initially synthesized as a large precursor protein which subsequently undergoes proteolytic cleavage at a cluster of basic residues approximately 110-140 amino acids from the C-terminus. The C-terminal regions, or mature regions, of the proteins are all structurally related and the different family

members can be classified into distinct subgroups based on the extent of their homology. Although the homologies within particular subgroups range from 70% to 90% amino acid sequence identity, the homologies between subgroups are significantly lower, generally ranging from only 20% to 50%. In each case, the active species appears to be a disulfide-linked dimer of C-terminal fragments. Studies have shown that when the pro-region of a member of the TGF-β family is coexpressed with a mature region of another member of the TGF-β family, intracellular dimerization and secretion of biologically active homodimers occur (Gray, A. *et al., Science*, 247:1328, 1990). Additional studies by Hammonds, *et al.*, (*Molec. Endocrin.* 5:149, 1991) showed that the use of the BMP-2 pro-region combined with the BMP-4 mature region led to dramatically improved expression of mature BMP-4. For most of the family members that have been studied, the homodimeric species has been found to be biologically active, but for other family members, like the inhibins (Ling, *et al., Nature*, 321 :779, 1986) and the TGF-βs (Cheifetz, *et al., Cell*, 48:409, 1987), heterodimers have also been detected, and these appear to have different biological properties than the respective homodimers.

The study of receptor-ligand interactions has revealed a great deal of information about how cells respond to external stimuli. This knowledge has led to the development of therapeutically important compounds, such as erythropoietin, colony stimulating factors and PDGF.

Summary of the Invention

The present invention provides receptors for the growth differentiation factor (GDF) growth factor family. These receptors are useful for identifying antagonists and agonists for agricultural and human therapeutic purposes.

In a first embodiment, the invention provides a recombinant cell line that expresses growth differentiation factor-8 (GDF-8) or growth differentiation factor-11 (GDF-11) receptor polypeptide. Also included are antibodies that bind to GDF receptors, polynucleotides encoding the receptors and the GDF receptor proteins themselves.

from a GDF-receptor gene, whereby the binding of the antisense to the mRNA segment inhibits GDF-receptor expression.

Brief Description of the Figures

Figure 1a and 1b are the nucleotide and amino acid sequence of murine GDF-8.

Figure 1c and 1d are the nucleotide and amino acid sequence of human GDF-8.

Figures 2a-2e are the nucleotide and amino acid sequence of baboon, bovine, chicken, rat, and turkey GDF-8.

Figures 3a and 3b are Northern blots showing expression of GDF-8 in muscle and in various species, respectively.

Figures 4a and 4b show the nucleotide and amino acid sequence of murine GDF-11 and expression of GDF-11, respectively.

Figure 5 shows an autoradiogram showing GDF-8.

Figures 6 and 7 show binding studies for GDF-8.

Figures 8-11 show 4 myoblast cell lines that do not bind GDF-8.

Figure 12 shows the construction of GDF-11 null mice by homologous targeting. a) is a map of the GDF-11 locus (top line) and targeting construct (second line). The black and stippled boxes represent coding sequences for the pro-and C-terminal regions, respectively. The targeting construct contains a total of 11 kb of homology with the GDF-11 gene. A probe derived from the region upstream of the 3' homology fragment and downstream of the first EcoRI site shown hybridizes to a 6.5 kb EcoR1 fragment in the GDF-11 gene and a 4.8 kb fragment in a homologously targeted gene. Abbreviations: X, Xba1; E, EcoR1. b) Geneomic Southern of DNA prepared from F1 heterozygous mutant mice (lanes 1 and 2) and offspring derived from a mating of these mice (lanes 3-12).

Figure 13 shows kidney abnormalities in GDF-11 knockout mice. Kidneys of newborn animals were examined and classified according to the number of normal sized or small kidneys as shown at the top. Numbers in the table indicate number of animals falling into each classification according to genotype.

The polynucleotide encoding GDF receptors for GDFs such as GDF-8 or 11 (shown in the figures). When the sequence is RNA, the deoxyribonucleotides A, G, C, and T are replaced by ribonucleotides A, G, C, and U, respectively. Also included in the invention are fragments (portions) of the above-described nucleic acid sequences that are at least 15 bases in length, which is sufficient to permit the fragment to selectively hybridize to DNA that encodes the GDF receptor. "Selective hybridization" as used herein refers to hybridization under moderately stringent or highly stringent physiological conditions (See, for example, the techniques described in Maniatis et al., 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., incorporated herein by reference), which distinguishes related from unrelated nucleotide sequences.

In nucleic acid hybridization reactions, the conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, GC v. AT content), and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. An additional consideration is whether one of the nucleic acids is immobilized, for example, on a filter.

An example of progressively higher stringency conditions is as follows: 2 x SSC/0.1% SDS at about room temperature (hybridization conditions); 0.2 x SSC/0.1% SDS at about room temperature (low stringency conditions); 0.2 x SSC/0.1% SDS at about 42°C (moderate stringency conditions); and 0.1 x SSC at about 68°C (high stringency conditions). Washing can be carried out using only one of these conditions, *e.g.*, high stringency conditions, or each of the conditions can be used, *e.g.*, for 10-15 minutes each, in the order listed above, repeating any or all of the steps listed. However, as mentioned above, optimal conditions will vary, depending on the particular hybridization reaction involved, and can be determined empirically.

hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al., Nucl. Acid Res.*, 9:879, 1981). Alternatively, a subtractive library, as illustrated herein is useful for elimination of non-specific cDNA clones.

When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the synthesis of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid- or phage-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al., Nucl. Acid Res.*, 11:2325, 1983).

A cDNA expression library, such as lambda gt11, can be screened indirectly for GDF receptors peptides having at least one epitope, using antibodies specific for GDF receptors. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of GDF receptors cDNA.

Alterations in GDF receptors nucleic acid include intragenic mutations (*e.g.*, point mutation, nonsense (stop), missense, splice site and frameshift) and heterozygous or homozygous deletions. Detection of such alterations can be done by standard methods known to those of skill in the art including sequence analysis, Southern blot analysis, PCR based analyses (*e.g.*, multiplex PCR, sequence tagged sites (STSs)) and *in situ*

vectors capable of expression and replication in a host are known in the art. Such vectors are used to incorporate DNA sequences of the invention.

Methods which are well known to those skilled in the art can be used to construct expression vectors containing the GDF receptors coding sequence and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic techniques. (See, for example, the techniques described in Maniatis et al., 1989 Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y.)

A variety of host-expression vector systems may be utilized to express the GDF receptors coding sequence. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing the GDF receptors coding sequence; yeast transformed with recombinant yeast expression vectors containing the GDF receptors coding sequence; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing the GDF receptors coding sequence; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing the GDF receptors coding sequence; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, retroviruses, adenovirus, vaccinia virus) containing the GDF receptors coding sequence, or transformed animal cell systems engineered for stable expression. Since GDF receptors has not been confirmed to contain carbohydrates,both bacterial expression systems as well as those that provide for translational and post-translational modifications may be used; *e.g.*, mammalian, insect, yeast or plant expression systems.

Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, transcription enhancer elements, transcription terminators, etc. may be used in the expression vector (see *e.g.*, Bitter et al., 1987, Methods in Enzymology 153:516-544). For example, when

Mammalian cell systems which utilize recombinant viruses or viral elements to direct expression may be engineered. For example, when using adenovirus expression vectors, the GDF receptors coding sequence may be ligated to an adenovirus transcription/-translation control complex, *e.g.*, the late promoter and tripartite leader sequence. Alternatively, the vaccinia virus 7.5K promoter may be used. (*e.g.*, see, Mackett et al., 1982, Proc. Natl. Acad. Sci. USA 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl. Acad. Sci. USA 79: 4927-4931). Of particular interest are vectors based on bovine papilloma virus which have the ability to replicate as extrachromosomal elements (Sarver, et al., 1981, Mol. Cell. Biol. 1: 486). Shortly after entry of this DNA into mouse cells, the plasmid replicates to about 100 to 200 copies per cell. Transcription of the inserted cDNA does not require integration of the plasmid into the host's chromosome, thereby yielding a high level of expression. These vectors can be used for stable expression by including a selectable marker in the plasmid, such as, for example, the neo gene. Alternatively, the retroviral genome can be modified for use as a vector capable of introducing and directing the expression of the GDF receptors gene in host cells (Cone & Mulligan, 1984, *Proc. Natl. Acad. Sci. USA* 81:6349-6353). High level expression may also be achieved using inducible promoters, including, but not limited to, the metallothionine IIA promoter and heat shock promoters.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the GDF receptors cDNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. For example, following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine

Cell Lines

In one embodiment, the present invention relates to stable recombinant cell lines, the cells of which express GDF receptor polypeptides and contain DNA that encodes GDF receptors. Suitable cell types include but are not limited to cells of the following types: NIH 3T3 (Murine), C2C12, L6, and P19. C2C12 and L6 myoblasts will differentiate spontaneously in culture and form myotubes depending on the particular growth conditions (Yaffe and Saxel, 1977; Yaffe, 1968). P19 is an embryonal carcinoma cell line. Such cells are described, for example, in the Cell Line Catalog of the American Type Culture Collection (ATCC). These cells can be stably transformed by a method known to the skilled artisan. See, for example, Ausubel *et al.*, *Introduction of DNA Into Mammalian Cells*, in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, sections 9.5.1-9.5.6 (John Wiley & Sons, Inc. 1995). "Stable" transformation in the context of the invention means that the cells are immortal to the extent of having gone through at least 50 divisions.

GDF receptors can be expressed using inducible or constituitive regulatory elements for such expression. Commonly used constituitive or inducible promoters, for example, are known in the art. The desired protein encoding sequence and an operably linked promoter may be introduced into a recipient cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the desired molecule may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome. Therefore the cells can be transformed stably or transiently.

An example of a vector that may be employed is one which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector.

cDNA expression vectors incorporating such elements include those described by Okayama, H., Mol. Cell. Biol., 3:280 (1983), and others.

Once the vector or DNA sequence containing the construct has been prepared for expression, the DNA construct may be introduced (transformed) into an appropriate host. Various techniques may be employed, such as protoplast fusion, calcium phosphate precipitation, electroporation or other conventional techniques.

TRANSGENIC ANIMALS

In another embodiment, the present invention relates to transgenic animals having cells that express GDF receptors. Such transgenic animals, for example those containing the GDF-8 receptor, may have decreased fat content and increased muscle mass. The subject invention provides non-human transgenic animals which are useful as a source of food products with high muscle and protein content, and reduced fat and cholesterol content. The animals have been altered chromosomally in their germ cells and somatic cells so that the production of GDF-8 may be at "normal" levels, however, the GDF-8 receptor is produced in reduced amounts, or is completely disrupted, resulting in animals with decreased binding of GDF-8 and higher than normal levels of muscle tissue, preferably without increased fat and/or cholesterol levels. Accordingly, the present invention also includes food products provided by the animals. Such food products have increased nutritional value because of the increase in muscle tissue. The transgenic non-human animals of the invention include bovine, porcine, ovine and avian animals, for example.

The subject invention also provides a method of producing animal food products having increased muscle content. The method includes modifying the genetic makeup of the germ cells of a pronuclear embryo of the animal, implanting the embryo into the oviduct of a pseudopregnant female thereby allowing the embryo to mature to full term progeny, testing the progeny for presence of the transgene to identify transgene-positive progeny, cross-breeding transgene-positive progeny to obtain further transgene-positive progeny and processing the progeny to obtain foodstuff. The modification of the germ cell

The term "transgenic animal" also includes a "germ cell line" transgenic animal. A germ cell line transgenic animal is a transgenic animal in which the genetic information has been taken up and incorporated into a germ line cell, therefore conferring the ability to transfer the information to offspring. If such offspring in fact possess some or all of that information, then they, too, are transgenic animals.

The cDNA that encodes GDF receptors can be fused in proper reading frame under the transcriptional and translational control of a vector to produce a genetic construct that is then amplified, for example, by preparation in a bacterial vector, according to conventional methods. See, for example, the standard work: Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor Press 1989), the contents of which are incorporated by reference. The amplified construct is thereafter excised from the vector and purified for use in producing transgenic animals.

The term "transgenic" as used herein additionally includes any organism whose genome has been altered by *in vitro* manipulation of the early embryo or fertilized egg or by any transgenic technology to induce a specific gene knockout. The term "gene knockout" as used herein, refers to the targeted disruption of a gene *in vivo* with complete loss of function that has been achieved by any transgenic technology familiar to those in the art. In one embodiment, transgenic animals having gene knockouts are those in which the target gene has been rendered nonfunctional by an insertion targeted to the gene to be rendered non-functional by homologous recombination. As used herein, the term "transgenic" includes any transgenic technology familiar to those in the art which can produce an organism carrying an introduced transgene or one in which an endogenous gene has been rendered non-functional or "knocked out."

The transgene to be used in the practice of the subject invention may be a DNA sequence comprising a modified GDF receptors coding sequence. In a preferred embodiment, the GDF receptor gene is disrupted by homologous targeting in embryonic stem cells. For example, the entire mature C-terminal region of the GDF receptors gene may be deleted

hours is preferred. The GDF-8 antibody may be formulated in a formulation suitable for administration to a patient. Such formulations are known in the art.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of the GDF-8 receptor protein, *e.g.* amount of tissue desired to be formed, the site of tissue damage, the condition of the damaged tissue, the size of a wound, type of damaged tissue, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the types of agent, such as anti-GDF-8 receptor antibodies, to be used in the composition. Generally, systemic or injectable administration, such as intravenous (IV), intramuscular (IM) or subcutaneous (Sub-Q) injection. Administration will generally be initiated at a dose which is minimally effective, and the dose will be increased over a preselected time course until a positive effect is observed. Subsequently, incremental increases in dosage will be made limiting such incremental increases to such levels that produce a corresponding increase in effect, while taking into account any adverse affects that may appear. The addition of other known growth factors, such as IGF I (insulin like growth factor I), human, bovine, or chicken growth hormone which may aid in increasing muscle mass, to the final composition, may also affect the dosage. In the embodiment where an anti-GDF-8 receptor antibody is administered, the anti-GDF-8 antibody is generally administered within a dose range of about 0.1 ug/kg to about 100 mg/kg.; more preferably between about 10 mg/kg to 50 mg/kg.

The preparation of polyclonal antibodies is well-known to those skilled in the art. See, for example, Green *et al.*, *Production of Polyclonal Antisera*, in IMMUNOCHEMICAL PROTOCOLS (Manson, ed.), pages 1-5 (Humana Press 1992); Coligan *et al.*, *Production of Polyclonal Antisera in Rabbits, Rats, Mice and Hamsters*, in CURRENT PROTOCOLS IN IMMUNOLOGY, section 2.4.1 (1992), which are hereby incorporated by reference.

Therapeutic applications for antibodies disclosed herein are also part of the present invention. For example, antibodies of the present invention may also be derived from subhuman primate antibody. General techniques for raising therapeutically useful antibodies in baboons can be found, for example, in Goldenberg *et al.*, International Patent Publication WO 91/11465 (1991) and Losman *et al.*, *Int. J. Cancer* 46:310 (1990), which are hereby incorporated by reference.

Alternatively, a therapeutically useful anti-GDF receptors antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementarity determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain, and then substituting human residues in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA* 86:3833 (1989), which is hereby incorporated in its entirety by reference. Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature* 321: 522 (1986); Riechmann *et al.*, *Nature* 332: 323 (1988); Verhoeyen *et al.*, *Science* 239: 1534 (1988); Carter *et al.*, *Proc. Nat'l Acad. Sci. USA* 89: 4285 (1992); Sandhu, *Crit. Rev. Biotech.* 12: 437 (1992); and Singer *et al.*, *J. Immunol.* 150: 2844 (1993), which are hereby incorporated by reference.

Antibodies of the invention also may be derived from human antibody fragments isolated from a combinatorial immunoglobulin library. See, for example, Barbas *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 119 (1991); Winter *et al.*, *Ann. Rev. Immunol.* 12: 433 (1994), which are hereby incorporated by reference. Cloning and expression vectors that are useful for producing a human immunoglobulin phage library can be obtained, for example, from STRATAGENE Cloning Systems (La Jolla, CA).

enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association may be noncovalent, as described in Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA* 69:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. *See, e.g.*, Sandhu, *supra*. Preferably, the Fv fragments comprise V_H and V_L chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains connected by an oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by Whitlow *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 97 (1991); Bird *et al.*, *Science* 242:423-426 (1988); Ladner *et al.*, U.S. patent No. 4,946,778; Pack *et al.*, *Bio/Technology* 11: 1271-77 (1993); and Sandhu, *supra*.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick *et al.*, METHODS: A COMPANION TO METHODS IN ENZYMOLOGY, VOL. 2, page 106 (1991).

IDENTIFICATION OF GDF RECEPTORS

In another embodiment, the invention provides a method for identifying a GDF receptor polypeptide comprising incubating components comprising GDF polypeptide and a cell expressing a receptor or a soluble receptor under conditions sufficient to allow the GDF

Variants useful for the present invention comprise analogs, homologs, muteins and mimetics of GDF receptors that retain the ability to bind to their respective GDFs. Peptides of the GDF receptors refer to portions of the amino acid sequence of GDF receptors that also retain this ability. The variants can be generated directly from GDF receptors itself by chemical modification, by proteolytic enzyme digestion, or by combinations thereof. Additionally, genetic engineering techniques, as well as methods of synthesizing polypeptides directly from amino acid residues, can be employed.

Peptides of the invention can be synthesized by such commonly used methods as t-BOC or FMOC protection of alpha-amino groups. Both methods involve stepwise syntheses whereby a single amino acid is added at each step starting from the C terminus of the peptide (See, Coligan, *et al., Current Protocols in Immunology,* Wiley Interscience, 1991, Unit 9). Peptides of the invention can also be synthesized by the well known solid phase peptide synthesis methods described Merrifield, *J. Am. Chem. Soc.*, 85:2149, 1962), and Stewart and Young, *Solid Phase Peptides Synthesis*, (Freeman, San Francisco, 1969, pp.27-62), using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 1/4-1 hours at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material. This can normally be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and quantitated by the solid phase Edman degradation.

Alternatively, peptides can be produced by recombinant methods as described below.

are disclosed in PROTEIN ENGINEERING, *loc. cit.*, and CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, *supra.*

GDF RECEPTOR-BINDING AND BLOCKING AGENTS

In yet another embodiment, the present invention relates to GDF receptor-binding agents that block binding of GDFs to their receptors. Such agents could represent research and diagnostic tools in the study of muscle wasting disorder as described above and the development of more effective therapeutics. In addition, pharmaceutical compositions comprising GDF receptor-binding agents may represent effective therapeutics. In the context of the invention, the phrase "GDF receptor-binding agent" denotes a naturally occurring ligand of GDF receptors such as, for example: GDF-1-16; a synthetic ligand of GDF receptors, or appropriate derivatives of the natural or synthetic ligands. The determination and isolation of ligands is well described in the art. *See, e.g.*, Lerner, *Trends NeuroSci.* 17:142-146 (1994) which is hereby incorporated in its entirety by reference.

In yet another embodiment, the present invention relates to GDF receptor-binding agents that interfere with binding between GDF receptor and a GDF. Such binding agents may interfere by competitive inhibition, by non-competitive inhibition or by uncompetitive inhibition. Interference with normal binding between GDF receptors and one or more GDF can result in a useful pharmacological effect.

SCREEN FOR BINDING AND BLOCKING COMPOSITIONS

In another embodiment, the invention provides a method for identifying a composition which binds to GDF receptors. The method includes incubating components comprising the composition and GDF receptors under conditions sufficient to allow the components to interact and measuring the binding of the composition to GDF receptors. Compositions that bind to GDF receptors include peptides, peptidomimetics, polypeptides, chemical compounds and biologic agents as described above.

of the present invention can be extracted and purified from the culture media or a cell by using known protein purification techniques commonly employed, such as extraction, precipitation, ion exchange chromatography, affinity chromatography, gel filtration and the like. Compositions can be isolated by affinity chromatography using the modified receptor protein extracellular domain bound to a column matrix or by heparin chromatography.

Also included in the screening method of the invention is combinatorial chemistry methods for identifying chemical compounds that bind to GDF receptors. Thus, the screening method is also useful for identifying variants, binding or blocking agents, etc., which functionally, if not physically (*e.g.*, sterically) act as antagonists or agonists, as desired.

EXAMPLES

Distribution of receptors for GDF-8 and GDF-11.

The purified GDF-8 and GDF-11 proteins will be used primarily to assay for biological activities. In order to identify potential target cells for GDF-8 and GDF-11 action cells expressing their receptors will be searched. For this purpose, the purified protein will be radioiodinated using the chloramine T method, which has been used successfully to label other members of this superfamily, like TGF-ß (Cheifetz et al., 1987), activins (Sugino et al., 1988), and BMPs (Paralkar et al., 1991), for receptor-binding studies. The mature processed forms of GDF-8 and GDF-11 each contain multiple tyrosine residues. Two different approaches will then be taken to attempt to identify receptors for these proteins.

One approach will be taken to determine the number, affinity, and distribution of receptors. Either whole cells grown in culture, frozen sections of embryos or adult tissues, or total membrane fractions prepared from tissues or cultured cells will be incubated with the labeled protein, and the amount or distribution of bound protein will be determined. For experiments involving cell lines or membranes, the amount of

and their relative affinities. These studies will be critical as they will give an indication as to whether the molecules signal through the same or different receptors. Competition experiments using other TGF-ß family members will be performed to determine specificity. Some of these ligands are available commercially, and some others are available from Genetics Institute, Inc.

For these experiments, a variety of embryonic and adult tissues and cell lines will be tested. Based on the specific expression of GDF-8 in skeletal muscle and the phenotype of GDF-8 knock-out mice, initial studies focus on embryonic and adult muscle tissue for membrane preparation and for receptor studies using frozen sections. In addition, myoblasts will be isolated and cultured from embryos at various days of gestation or satellite cells from adult muscle as described (Vivarelli and Cossu, 1986; Cossu et al., 1980). The binding studies on these primary cells after various days in culture will be performed and binding sites localized by autoradiography so that the binding sites can be co-localized with various myogenic markers, such as muscle myosin (Vivarelli et al., 1988), and correlate binding with the differentiation state of the cells, such as formation of multinucleated myotubes. In addition to using primary cells, cell lines will be utilized to look for receptors. In particular, the initial focus will be on three cells lines, C2C12, L6, and P19. C2C12 and L6 myoblasts will differentiate spontaneously in culture and form myotubes depending on the particular growth conditions (Yaffe and Saxel, 1977; Yaffe, 1968). P19 embryonal carcinoma cells can be induced to differentiate into various cell types, including skeletal muscle cells in the presence of DMSO (Rudnicki and McBurney, 1987). Receptor binding studies will be carried out on these cell lines under various growth conditions and at various stages of differentiation.

Although the initial studies will focus on muscle cells, other tissues and cell types will be examined for the presence of GDF-8 and GDF-11 receptors.

Recombinant human GDF-8 homodimer was used in these binding studies. The rh-GDF-8 was expressed using CHO cells and purified to approximately 90% purity. The

SV40 origin of replication. The library will be plated, and cells from each plate will be pooled into broth and frozen. Aliquots from each pool will then be grown for preparation of DNA. Each individual pool will be transiently transfected into COS cells in chamber slides, and transfected cells will be incubated with iodinated GDF-8 or GDF-11. After washing away the unbound protein, the sites of ligand binding will be visualized by autoradiography. Once a positive pool is identified, the cells from that pool will be replated at lower density, and the process will be repeated. Positive pools will then be plated, and individual colonies will be picked into grids and re-analyzed as described (Wong et al., 1985).

We will attempt to carry out this screen initially using pool sizes of 1500 colonies. In order to be certain that we will be able to identify a positive clone in a mixture of this complexity, we will carry out a control experiment using TGF-ß and a cloned type II receptor. The coding sequence for the TGF-ß type II receptor will be cloned into the pcDNA-1 vector, and bacteria transformed with this construct will be mixed with bacteria from our library at various ratios, including 1:1500. We will then transfect DNA prepared from this mixture into COS cells, incubate with iodinated TGF-ß, and visualize by autoradiography. If we can see positive signals at a ratio of 1:1500, we will begin screening pools of 1500 clones. Otherwise, we will use smaller pool sizes corresponding to ratios at which the procedure is sensitive enough to identify a positive signal in our control experiments. While we have no previous experience in expression cloning per se, we have constructed over 50 cDNA libraries in the past, and many of these have yielded a high frequency of full-length cDNA clones.

We will also use a second parallel strategy to attempt to clone the GDF-8 and GDF-11 receptors. We will take advantage of the fact that most receptors for members of the TGF-ß superfamily that have been identified belong to the membrane-spanning serine/threonine kinase family (for review, see Massague, 1996). Because the cytoplasmic domains of these receptors are related in sequence, we will attempt to use degenerate PCR to clone members of this receptor family that are expressed in tissues

both type I and type II receptors are also involved in signalling GDF-8 and GDF-11. If so, it will be important to clone both of these receptor subtypes in order to fully understand how GDF-8 and GDF-11 transmit their signals. Because we cannot predict whether the type I receptor is capable of interacting with GDF-8 and GDF-11 in the absence of the type II receptor, we will focus first on cloning the type II receptor(s). Only after we have at least one type II receptor for these ligands in hand will we attempt to identify the type I receptors for GDF-8 and GDF-11. Our general strategy will be to co-transfect the type II receptor with each of the type I receptors that we identify in the PCR screen and then assay the transfected cells by crosslinking as described in Specific Aim 4. If the type I receptor is part of the receptor complex for GDF-8 or GDF-11, we should be able to detect two cross-linked receptor species in the transfected cells, one corresponding to the type I receptor and the other corresponding to the type II receptor.

The search for GDF-8 and GDF-11 receptors is further complicated by the fact at least one member of the TGF-ß superfamily, namely, GDNF, is capable of signalling through a completely different type of receptor complex involving a GPI-linked component (GDNFR-alpha) and a receptor tyrosine kinase (c-ret) (Trupp et al., 1996; Durbec et al., 1996; Treanor et al., 1996; Jing et al., 1996). Although GDNF is the most distantly-related member of the TGF-ß superfamily, it is certainly possible that other TGF-ß family members may also signal through an analogous receptor system. If GDF-8 and GDF-11 do signal through a similar receptor complex, our expression screening approach should be able to identify at least the GPI-linked component (indeed GDNFR-alpha was identified using an expression screening approach) of this complex. However, identifying the analogous receptor tyrosine kinase would probably require a substantial amount of additional work, such as biochemical purification of the complex. In the case of GDNF, the similar phenotypes of GDNF- and c-ret-deficient mice suggested c-ret as a potential receptor for GDNF.

but the lethality may have been related to the fact that the kidneys in homozygous mutants were either severely hypoplastic or completely absent. A summary of the kidney abnormalities in these mice is shown in Figure 13.

Anatomical Differences In Knockout Mice

Homozygous mutant animals were easily recognizable by their severely shortened or absent tails (Figure 14a). To further characterize the tail defects in these homozygous mutant animals, we examined their skeletons to determine the degree of disruption of the caudal vertebrae. A comparison of wild-type and mutant skeleton preparations of late stage embryos and newborn mice, however, revealed differences not only in the caudal region of the animals but in many other regions as well. In nearly every case where differences were noted, the abnormalities appeared to represent homeotic transformations of vertebral segments in which particular segments appeared to have a morphology typical of more anterior segments. These transformations, which are summarized in Figure 15, were evident throughout the axial skeleton extending from the cervical region to the caudal region. Except for the defects seen in the axial skeleton, the rest of the skeleton, such as the cranium and limb bones, appeared normal.

Anterior transformations of the vertebrae in mutant newborn animals were most readily apparent in the thoracic region, where there was a dramatic increase in the number of thoracic (T) segments. All wild-type mice examined showed the typical pattern of 13 thoracic vertebrae each with its associated pair of ribs (Figure 14(b,e)). In contrast, homozygous mutant mice showed a striking increase in the number of thoracic vertebrae. All homozygous mutants examined had 4 to 5 extra pairs of ribs for a total of 17 to 18 (Figure 14(d,g)) although in over 1/3 of these animals, the 18th rib appeared to be rudimentary. Hence, segments that would normally correspond to lumbar (L) segments L1 to L4 or L5 appeared to have been transformed into thoracic segments in mutant animals.

Transformations of the axial skeleton also extended into the lumbar region. Whereas wild-type animals normally have only 6 lumbar vertebrae, homozygous mutants had 8-9. At least 6 of the lumbar vertebrae in the mutants must have derived from segments that would normally have given rise to sacral and caudal vertebrae as the data described above suggest that 4 to 5 lumbar segments were transformed into thoracic segments. Hence, homozygous mutant mice had a total of 33-34 presacral vertebrae compared to 26 presacral vertebrae normally present in wild-type mice. The most common presacral vertebral patterns were C7/T18/L8 and C7/T18/L9 for mutant mice compared to C7/T13/L6 for wild-type mice. The presence of additional presacral vertebrae in mutant animals was obvious even without detailed examination of the skeletons as the position of the hindlimbs relative to the forelimbs was displaced posteriorly by 7-8 segments.

Although the sacral and caudal vertebrae were also affected in homozygous mutant mice, the exact nature of each transformation was not as readily identifiable. In wild-type mice, sacral segments S1 and S2 typically have broad transverse processes compared to S3 and S4. In the mutants, there did not appear to be an identifiable S1 or S2 vertebra. Instead, mutant animals had several vertebrae that appeared to have morphology similar to S3. In addition, the transverse processes of all 4 sacral vertebrae are normally fused to each other although in newborns often only fusions of the first 3 vertebrae are seen. In homozygous mutants, however, the transverse processes of the sacral vertebrae were usually unfused. In the caudalmost region, all mutant animals also had severely malformed vertebrae with extensive fusions of cartilage. Although the severity of the fusions made it difficult to count the total number of vertebrae in the caudal region, we were able to count up to 15 transverse processes in several animals. We were unable to determine whether these represented sacral or caudal vertebrae in the mutants because we could not establish morphologic criteria for distinguishing S4 from caudal vertebrae even in wild-type newborn animals. Regardless of their identities, the total number of vertebrae in this region was significantly reduced from the normal number of approximately 30. Hence, although the mutants had significantly more thoracic and

represented true transformations of segment identities rather than the insertion of additional segments, for example, by an enhanced rate of somitogenesis.

Alterations in expression of homeobox containing genes are known to cause transformations in Drosophila and in vertebrates. To see if the expression patterns of Hox genes (the vertebrate homeobox containing genes) were altered in GDF-11 null mutants we determined the expression pattern of 3 representative Hox genes, Hoxc-6, Hoxc-8 and Hoxc-11, in day 12.5 p.c. wild-type, heterozygous and homozygous mutant embryos by whole mount in situ hybridization. The expression pattern of Hoxc-6 in wild-type embryos spanned prevertebrae 8-15 which correspond to thoracic segments T1-T8. In homozygous mutants, however, the Hoxc-6 expression pattern was shifted posteriorly and expanded to prevertebrae 9-18 (T2-T11). A similar shift was seen with the Hoxc-8 probe. In wild-type embryos, Hoxc-8 was expressed in prevertebrae 13-18 (T6-T11) but, in homozygous mutant embryos, Hoxc-8 was expressed in prevertebrae 14-22 (T7-T15). Finally, Hoxc-11 expression was also shifted posteriorly in that the anterior boundary of expression changed from prevertebrae 28 tin wild-type embryos to prevertebrae 36 in mutant embryos. (Note that because the position of the hindlimb is also shifted posteriorly in mutant embryos, the Hoxc-11 expression patterns in wild-type and mutant appeared similar relative to the hindlimbs). These data provide further evidence that the skeletal abnormalities seen in mutant animals represent homeotic transformations.

The phenotype of GDF-11 mice suggested that GDF-11 acts early during embryogenesis as a global regulator of axial patterning. To begin to examine the mechanism by which GDF-11 exerts its effects, we determined the expression pattern of GDF-11 in early mouse embryos by whole mount in situ hybridization. At these stages the primary sites of GDF-11 expression correlated precisely with the known sites at which mesodermal cells are generated. Expression of GDF-11 was first detected at day 8.25-8.5 p.c. (8-10 somites) in the primitive streak region, which is the site at which ingressing cells form the mesoderm of the developing embryo. Expression was maintained in the primitive

may be, the fact that gross anterior/posterior patterning still does occur in GDF-11 knockout animals suggests that GDF-11 may not be the sole regulator of anterior/posterior specification. Nevertheless, it is clear that GDF-11 plays an important role as a global regulator of axial patterning and that further study of this molecule will lead to important new insights into how positional identity along the anterior/posterior axis is established in the vertebrate embryo.

Similar phenotypes are expected in GDF-8 knockout animals. For example, GDF-8 knockout animals are expected to have increased number of ribs, kidney defects and anatomical differences when compared to wild-type.

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What Is Claimed Is:

1. A recombinant cell line that expresses growth differentiation factor-8 (GDF-8) or growth differentiation factor-11 (GDF-11) receptor polypeptide.

2. The cell line of claim 1, wherein the cell is selected from the group of species consisting of avian, bovine, ovine, piscine, murine, human and porcine.

3. An antibody which specifically binds to growth differentiation factor-8 (GDF-8) receptor polypeptide or fragments thereof.

4. The antibody of claim 7, wherein the antibody is a monoclonal antibody.

5. An antibody which specifically binds to growth differentiation factor-11 (GDF-11) receptor polypeptide or fragments thereof.

6. The antibody of claim 7, wherein the antibody is a monoclonal antibody.

7. An isolated polynucleotide which encodes growth differentiation factor-8 (GDF-8) receptor.

8. An isolated polynucleotide which encodes growth differentiation factor-11 (GDF-11) receptor.

9. An expression vector containing in operable linkage the polynucleotide as in claim 7 or 8.

10. A host cell containing the vector of claim 9.

21. The method of claim 20, wherein the anti-GDF- antibody is administered within a dose range of 0.1 ug/kg to 100 mg/kg.

22. The method of claim 20, wherein the antibody is formulated in a pharmaceutically acceptable carrier.

23. A method for identifying a GDF receptor polypeptide comprising:

a) incubating components comprising GDF polypeptide and a cell expressing a receptor or a soluble receptor under conditions sufficient to allow the GDF to bind to the receptor;

b) measuring the binding of the GDF polypeptide to the receptor; and

c) isolating the receptor.

24. The method of claim 23, wherein the GDF is GDF-8 or GDF-11.

25. A method for identifying a compound which binds to GDF receptor polypeptide comprising:

a) incubating components comprising the compound and GDF polypeptide under conditions sufficient to allow the components to interact; and

b) measuring the binding or effect of binding of the compound to GDF receptor polypeptide.

26. The method of claim 25, wherein the compound is a peptide.

27. The method of claim 25, wherein the compound is a peptidomimetic.

28. The method of claim 25, wherein the GDF receptor is expressed in a cell.

29. The method of claim 28, wherein the cell is the cell of claim 1.

35. The method of claim 34, wherein the introducing of the transgene into the embryo is by introducing an embryonic stem cell containing the transgene into the embryo.

36. The method of claim 34, wherein the introducing of the transgene into the embryo is by infecting the embryo with a retrovirus containing the transgene.

37. A transgenic non-human animal having a transgene disrupting or interfering with expression of GDF-receptor chromosomally integrated into the germ cells of the animal.

38. The transgenic non-human animal of claim 37, wherein the transgene comprises GDF-receptor antisense polynucleotide.

39. A method for inhibiting the expression of GDF-receptor in a cell comprising contacting GDF-receptor with an inhibiting effective amount of an antisense oligonucleotide that binds to a segment of an mRNA transcribed from a GDF-receptor gene, whereby the binding of the antisense to the mRNA segment inhibits GDF-receptor expression.

40. Substantially pure GDF receptor polypeptide.

41. The GDF receptor of claim 40, wherein GDF is GDF-8 or GDF-11.

1261 AAGTCATGGAAGGTCTTCCCCTCAATTTCGAAGTGTGAATTCAAGCAGCACAGGCTGTA 1320
1321 GGGCTTGAGTATGCTCTAGTAAGGTAGGCAGAGCTACAGTGTATGAACTAAAAGAGAGA 1380
1381 ATAGATGGAATGGTTGGCATTCAAGCACGAAATAAAGCATACTATAGGATGTTGTATGA 1440
1441 TTTCCAGAGTTTTTGAAATAGATGGAGATCAAATTAGATTATGTCCATATATGTATATT 1500
1501 ACAACTACAATCTAGGCAAGGAAGTGAGAGCAGATCTTGTGGTCTGCTGAGTTAGGAGG 1560
1561 TATGATTAAAAGGTAAAGTCTTATTTCCTAACAGTTTCACTTAATATTTACAGAAGAATC 1620
1621 TATATGTAGGCTTTGTAAAGTGTAGGATTGTTATCATTTAAAAACATCATGTACACTTAT 1680
1681 ATTTGTATTGTATACTTGGTAAGATAAAATTCCACAAGTAGGAATGGGGCCTCACATAC 1740
1741 ACATTGCCATTCCTATTATAATTGGACAATCCACCAGGTCCTAATGCAGTGCTGAAATGG 1800
1801 CTCCTACTGGACCTCTCGATAGAACACTCTACAAAGTAGGAGTCTCTCTCTCCCTTCCAG 1860
1861 GTGCATCTCCAGCAGAGAGCAGCACTAAGTGTTCAATGCATTTGTTTAAGGAAAGAAGAAT 1920
1921 GTTTTTTTCTAGAGGTCAACTTTCAGTCAAGTCTTGCAGCGCCCAGTGACTGCTGCATC 1980
1981 TTAAAAGGCAGCAAACAGTATTCATTTTTTAATCTAAATTTCAAAATCACTGTCTGCCT 2040
2041 TTATCACATGGCAATTTGTGGTAAAATAATGGAAATGACTGGTTCTATCAATATTGTAT 2100
2101 AAAAGACTCTGAAGAATTACATTTATATAATATGTATACAATATTGTTTTGTAAATAAG 2160
2161 TGTCTCCTTTTATATTTACTTTGGTATATTTTTACACTAATGAAATTTCAAATCATTAAA 2220
2221 GTACAAAGACATGTCATGTATCACAAAAAGCTGACTGCTTCTATTTCAGAGTGAATTAC 2280
2281 CAGATTCAATAGTGGTCTTAAAACTCTGTATGTTAAGATTACAAGTTATATTACAATCA 2340
2341 ATTTATGTATTTTTTACATTATCAACTTATGGTTTCATGTGGCTGTATCTATGAATGTG 2400
2401 GCTCCCAGTCAAATTCAATGCCCACCATTTTAAAAATTACAGCATTACTAAACATAC 2460
2461 CAACATGTATCTAAAGAAATACAAATATGGTATCTCAATACAGCTACTTTTTTATTTTA 2520
2521 TAATTTGACAATGAATACATTTCTTTTATTTACTTCAGTTTTATAAATTGGAACTTTGTT 2580
2581 TATCAAATGTATTGTACTCATAGCTAAATGAAATTATTTCTTACATAAAAATGTGTACAA 2640
2641 ACTATAAATTAAAGTGTTTTCACATTTTTGAAAGGC 2676

FIG.1 b

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1201  GTTCATAACTTCCTAAAACATGGAAGGTTTTCCCCTCAACAATTTTGAAGCTGTGAAATT  1260
1261  AAGTACGACAGGCTATAGGCCTAGACTATGCTACAGTCACTTAACCATAAGCTACAGTAT  1320
1321  GTAAACTAAAACGGCGAATATATGCAATGGTTGCCATTTAACCATCCAAACAAATCATAC  1380
1381  AAGAAAGTTTTATGATTCCAGAGTTTTTGAGCTAGAAGGAGATCAAATTACATTTATGT  1440
1441  TCCTATATATTACAACATCGGCGAGCAAATGAAGCGATTCTCCTTGAGTTCTGATGAAT  1500
1501  TAAAGGAGTATCCTTTAAAGTCTATTTCTTTAAAGTTTTGTTTAATATTTACAGAAAAT  1560
1561  CCAGATACAGTATTGGTAAAATGCAGGATTGTTATATACCATCATTCGAATCATCCTTAA  1620
1621  ACACTTGAATTTATATTGTATGGTAGTATACTTGGTAAGATAAAATTCCACAAAAATACG  1680
1681  GATGGTGCAGCATATGCAATTTCGATTCCTATTATAATTGACACAGTACATTAACAATCC  1740
1741  ATGCCAACGGTGCTAATACGATACGCTGAATGTCTGAGCCTACCAGGTTTATCACATAAA  1800
1801  AAACATTCAGTAAAATACTAGTTTCTTTTCTTCAGGTGCATTTTCCTACACCTCCAA   1860
1861  ATCAGGAATGGATTTTCTTTAATGTAAGAAGAATCATTTTTCTAGAGGTTGGCTTTCAAT  1920
1921  TCTGTAGCATACTTGGAGAACTGCATTATCTTAAAAGCCAGTCAAATGGTGTTTGTTTT  1980
1981  TATCAAAATGTCAAAATAACATACTTGGAGAAGTATGTAATTTTGTCTTTGGAAAATTAC  2040
2041  AACACTGCCTTTGCAACACTGCAGTTTTTATGGTAAAATAATAGAAATGATCGACTCTAT  2100
2101  CAATATTGTATAAAAGACTGAACAATGCATTTATATAATATGTATACAATATTGTTTT  2160
2161  GTAAATAAGTGTCTCCTTTTTTATTTACTTTGGTATATTTTTACACTAAGGACATTTCAA  2220
2221  ATTAAGTACTAAGGCACAAGACATGTCATGCATCACAGAAAGCAACTACTTATATTTC  2280
2281  ACACCAAATTACCAGATTAAATAGTGGTCTTAAAACTCCATATGTTAATGATTAGATGGT  2340
2341  TATATTACAATCATTTTATATTTTTTACATGATTAACATTCACTTATGGATTCATGATG  2400
2401  GCTGTATAAGTGAATTTGAAATTTCAATGGTTTACTGTCATTGTGTTTAAATCTCAACC  2460
2461  TTCCATTATTTTAATACTTGCAAAAACATTACTAAGTATACCAAAATAATTGACTCTATT  2520
2521  ATCTGAAATGAAGAATAAACTGATGCTATCTCAACAATAACTGTTACTTTTATTTTATAA  2580
2581  TTTGATAATGAATATATTTCTGCATTTATTTACTTCTGTTTTGTAAATTGCGATTTTGTT  2640
2641  AATCAAATTTATTGTACTATGACTAAATGAAATTATTTCTTACATCTAATTTGTAGAAAC  2700
2701  AGTATAAGTTATATTAAAGTGTTTTCACATTTTTTTGAAAGAC  2743
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FIG. 1d

FIGURE 2b

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1/1                                                             31/11
ATG CAA AAA CTG CAA ATC TCT GTT TAT ATT TAC CTA TTT ATG CTG ATT GTT GCT GGC CCA
M   Q   K   L   Q   I   S   V   Y   I   Y   L   F   M   L   I   V   A   G   P
61/21                                                           91/31
GTG GAT CTG AAT GAG AAC AGC GAG CAG AAG GAA AAT GTG GAA AAA GAG GGC CTG TGT AAT
V   D   L   N   E   N   S   E   Q   K   E   N   V   E   K   E   G   L   C   N
121/41                                                          151/51
GGA TGT TTG TGG AGG GAA AAC ACT ACA TCC TCA AGA CTA GAA GCC ATA AAA ATC CAA ATC
G   C   L   W   R   E   N   T   T   S   S   R   L   E   A   I   K   I   Q   I
181/61                                                          211/71
CTC AGT AAA CTT CGC CTG GAA ACA GCT CCT AAC ATC AGC AAA GAT GCT ATC AGA CAA CTT
L   S   K   L   R   L   E   T   A   P   N   I   S   K   D   A   I   R   Q   L
241/81                                                          271/91
TTG CCC AAG GCT CCT CCA CTC CTG GAA CTG ATT GAT CAG TTC GAT GTC CAG AGA GAT GCC
L   P   K   A   P   P   L   L   E   L   I   D   Q   F   D   V   Q   R   D   A
301/101                                                         331/111
AGC AGT GAC GGC TCC TTG GAA GAC GAT GAC TAC CAC GCC AGG ACG GAA ACG GTC ATT ACC
S   S   D   G   S   L   E   D   D   D   Y   H   A   R   T   E   T   V   I   T
361/121                                                         391/131
ATG CCC ACG GAG TCT GAT CTT CTA ACG CAA GTG GAA GGA AAA CCC AAA TGT TGC TTC TTT
M   P   T   E   S   D   L   L   T   Q   V   E   G   K   P   K   C   C   F   F
421/141                                                         451/151
AAA TTT AGC TCT AAG ATA CAA TAC AAT AAA CTA GTA AAG GCC CAA CTG TGG ATA TAT CTG
K   F   S   S   K   I   Q   Y   N   K   L   V   K   A   Q   L   W   I   Y   L
481/161                                                         511/171
AGG CCT GTC AAG ACT CCT GCG ACA GTG TTT GTG CAA ATC CTG AGA CTC ATC AAA CCC ATG
R   P   V   E   T   P   A   T   V   F   V   Q   I   L   R   L   I   K   P   M
541/181                                                         571/191
AAA GAC GGT ACA AGG TAT ACT GGA ATC CGA TCT CTG AAA CTT GAC ATG AAC CCA GGC ACT
K   D   G   T   R   Y   T   G   I   R   S   L   K   L   D   M   N   P   G   T
601/201                                                         631/211
GGT ATT TGG CAG AGC ATT GAT GTG AAG ACA GTG TTG CAG AAC TGG CTC AAA CAA CCT GAA
G   I   W   Q   S   I   D   V   K   T   V   L   Q   N   W   L   K   Q   P   E
661/221                                                         691/231
TCC AAC TTA GGC ATT GAA ATC AAA GCT TTA GAT GAG AAT GGC CAT GAT CTT GCT GTA ACC
S   N   L   G   I   E   I   K   A   L   D   E   N   G   H   D   L   A   V   T
721/241                                                         751/251
TTC CCA GAA CCA GGA GAA GAT GGA CTG ACT CCT TTT TTA GAA GTC AAG GTA ACA GAC ACA
F   P   E   P   G   E   D   G   L   T   P   F   L   E   V   K   V   T   D   T
781/261                                                         811/271
CCA AAA AGA TCT AGG AGA GAT TTT GGG CTT GAT TGT GAT GAA CAC TCC ACA GAA TCT CGA
P   K   R   S   R   R   D   F   G   L   D   C   D   E   H   S   T   E   S   R
841/281                                                         871/291
TGC TGT CGT TAC CCT CTA ACT GTG GAT TTT GAA GCT TTT GGA TGG GAT TGG ATT ATT GCA
C   C   R   Y   P   L   T   V   D   F   E   A   F   G   W   D   W   I   I   A
901/301                                                         931/311
CCT AAA AGA TAT AAG GCC AAT TAC TGC TCT GGA GAA TGT GAA TTT GTA TTT TTG CAA AAG
P   K   R   Y   K   A   N   Y   C   S   G   E   C   E   F   V   F   L   Q   K
961/321                                                         991/331
TAT CCT CAT ACC CAT CTT GTG CAC CAA GCA AAC CCC AGA GGT TCA GCC GGC CCC TGC TGT
Y   P   H   T   H   L   V   H   Q   A   N   P   R   G   S   A   G   P   C   C
1021/341                                                        1051/351
ACT CCT ACA AAG ATG TCT CCA ATT AAT ATG CTA TAT TTT AAT GGC GAA GGA CAA ATA ATA
T   P   T   K   M   S   P   I   N   M   L   Y   F   N   G   E   G   Q   I   I
1081/351                                                        1111/371
TAC GGG AAG ATT CCA GGC ATG GTA GTA GAT CGC TGT GGC TGT TCA TCA
Y   G   K   I   P   A   M   V   V   D   R   C   G   C   S   *
```

Bovine CDF-3

FIGURE 2d

```
1/1                                                                 31/11
ATG ATT CAA AGA CCC CAA ATC TAT GTT TAT ATT TAC CTG TTT GTG CTG ATT GCT GCT GCC
M   I   Q   R   P   Q   M   Y   V   Y   I   Y   L   F   V   L   I   A   A   G
61/21                                                               91/31
CCA GTG GAT CTA AAT GAG GAC ACT GAG AGA GAG GCG AAT GTC GAA AAA GAG GGG CTG TGT
P   V   D   L   N   E   D   S   E   R   E   A   N   V   E   K   E   G   L   C
121/41                                                              151/51
AAT GGG TGT GGG TGG AGA CAA AAC AGA AGC TAC TCC AGA ATA GAA GGC ATA AAA ATT CAA
N   A   C   A   W   R   Q   N   T   R   Y   S   R   I   E   A   I   K   I   Q
181/61                                                              211/71
ATC CTG AGT AAA CTC CGG CTG GAA ACA GCG CCT AAC ATC AGC AAA GAT GCT ATA AGA CAA
I   L   S   K   L   R   L   E   T   A   P   N   I   S   K   D   A   I   R   Q
241/81                                                              271/91
CTT CTG CCC AGA GCG CCT CCA CTC CGG GAA CTG ATC GAT CAG TAC GAC GTC CAG AGG GAT
L   L   P   R   A   P   P   L   R   E   L   I   D   Q   Y   D   V   Q   R   D
301/101                                                             331/111
GAC AGC AGT GAC CGC TCT TTG GAA GAT GAC GAT TAT CAC GCT ACC ACG GAA ACA ATC ATT
D   S   S   D   R   S   L   E   D   D   D   Y   H   A   T   T   E   T   I   I
361/121                                                             391/131
ACC ATG CCT ACC GAG TCT GAC TTT CTA ATG CAA GCG GAT GGA AAG CCC AAA TGT TGC TTT
T   M   P   T   E   S   D   F   L   M   Q   A   D   G   K   P   K   C   C   F
421/141                                                             451/151
TTT AAA TTT AGC TCT AAA ATA CAG TAC AAC AAA GTG GTA AAG GCC CAG CTG TGG ATA TAT
F   K   F   S   S   K   I   Q   Y   N   K   V   V   K   A   Q   L   W   I   Y
481/161                                                             511/171
CTG AGA CCC GTC AAG ACT CCT AGA AGA GTG TTT GTG CAA ATC CTG AGA CTC ATC AAA CCC
L   R   A   V   K   T   P   T   T   V   F   V   Q   I   L   R   L   I   K   P
541/181                                                             571/191
ATG AAA GAC GGT ACA AGG TAT ACC GGA ATC CGA TCT CTG AAA CTT GAC ATG AGC CCA GGC
M   K   D   G   T   R   Y   T   G   I   R   S   L   K   L   D   M   S   P   G
601/201                                                             631/211
ACT GGT ATT TGG CAG AGT ATT GAT GTG AAG ACA GTG TTG GAA AAT TGG CTC AAA CAG CCT
T   G   I   W   Q   S   I   D   V   K   T   V   L   E   N   W   L   K   Q   P
661/221                                                             691/231
GAA TCC AAC TTA GGC ATT GAA ATC AAA GCT TTG GAT GAG AAT GGG CAT GAT CTT GCT GTA
E   S   N   L   G   I   E   I   K   A   L   D   E   N   G   H   D   L   A   V
721/241                                                             751/251
ACC TTC CCA GGA CCA GGA GAA GAT GGG CTG AAT CCC TTT TTA GAA GTC AAA GTA ACA GAC
T   F   P   G   P   G   E   D   G   L   N   P   F   L   E   V   K   V   T   D
781/261                                                             811/271
ACA CCC AAG AGG TCC CGG AGA AAC TTT GGG CTT GAC TGT GAT GAA CAC TCC ACG GAA TCC
T   P   K   R   S   R   R   D   F   G   L   D   C   D   E   H   S   T   E   S
841/281                                                             871/291
CGG TGC TGT CGC TAC CCC CTC ACG GTC GAT TTC GAA GCC TTT GGA TGG GAC TGG ATT ATT
R   C   C   R   Y   P   L   T   V   D   F   E   A   F   G   W   D   W   I   I
901/301                                                             931/311
GCA CCC AAA AGA TAT AAG GCT AAT TAC TGC TCT GGA GAG TGT GAA TTT GTG TTC TTA CAA
A   P   K   R   Y   K   A   N   Y   C   S   G   E   C   E   F   V   F   L   Q
961/321                                                             991/331
AAA TAT CCG CAT ACT CAT CTT GTG CAC CAA GCA AAC CCC AGA GGC TCG GCA GGC CCT TGC
K   Y   P   H   T   H   L   V   H   Q   A   N   P   R   G   S   A   G   P   C
1021/341                                                            1051/351
TGC ACG CCA ACA AAA ATG TCT CCC ATT AAT ATG CTA TAT TTT AAT GGC AAA GAA CAA ATA
C   T   P   T   K   M   S   P   I   N   M   L   Y   F   N   G   K   E   Q   I
1081/361                                                            1111/371
ATA TAT GGG AAA ATT CCA GGC ATG GTA GTA GAC CGG TGT GGG TGC TCC TCA
I   Y   G   K   I   P   A   M   V   V   D   R   C   G   C   S
```

Rat GDF-8

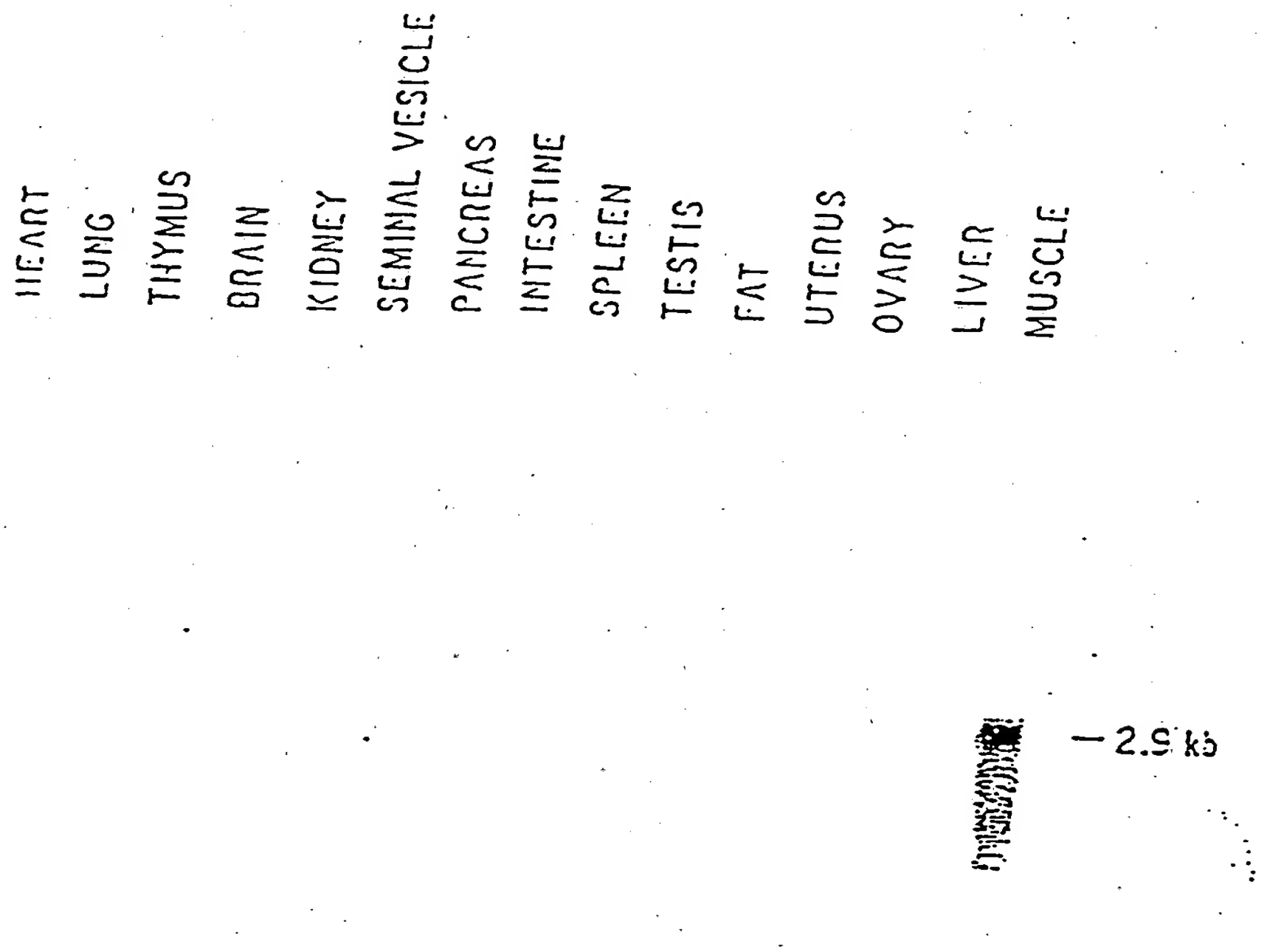

HEART
LUNG
THYMUS
BRAIN
KIDNEY
SEMINAL VESICLE
PANCREAS
INTESTINE
SPLEEN
TESTIS
FAT
UTERUS
OVARY
LIVER
MUSCLE
2.9 kb

FIG. 3a

GDF-8

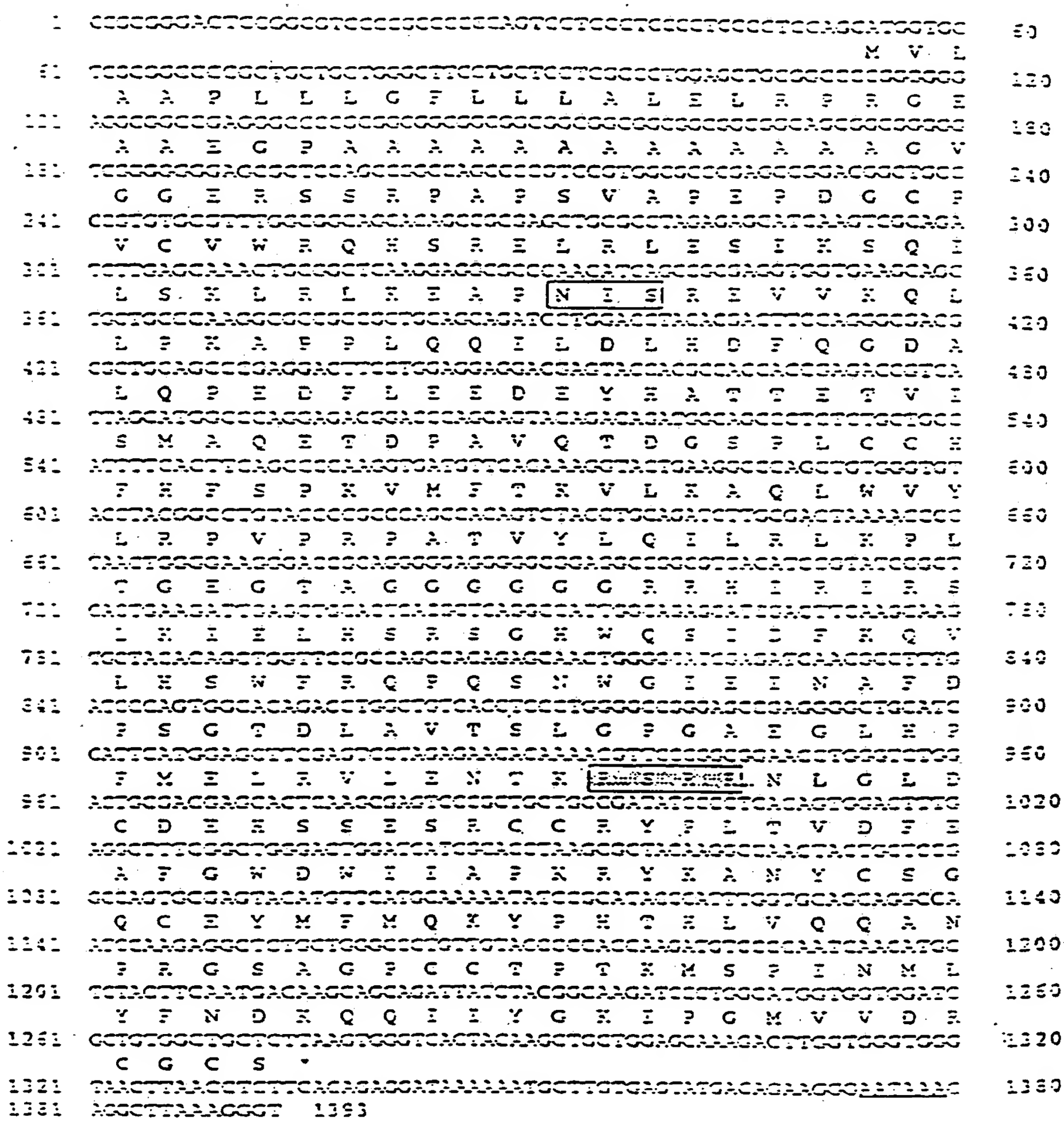


FIGURE 4A

GDF-11

CHO hGDF-8

Figure 5

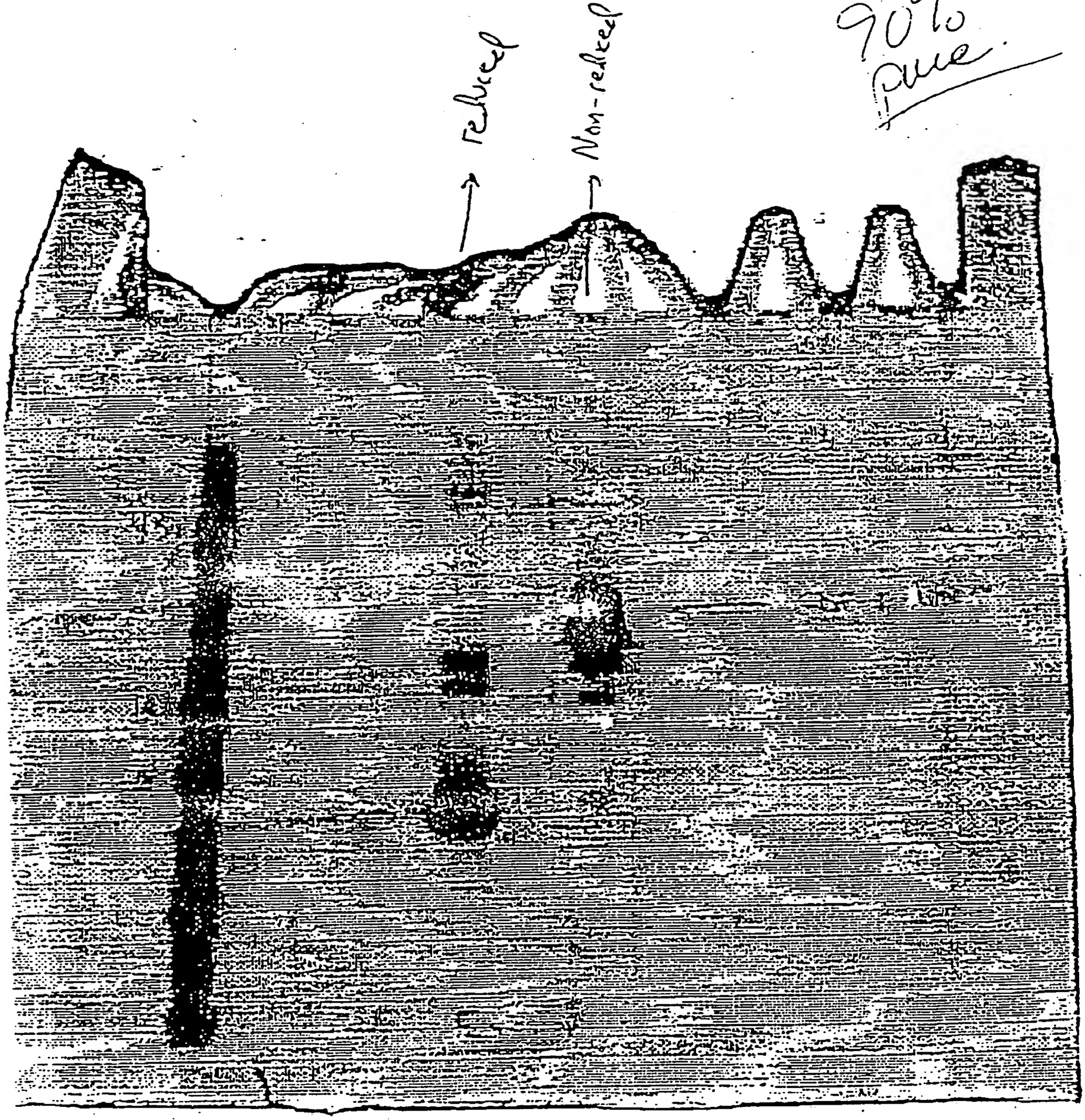


Figure 7

GDF-8 Binding to G8 Myoblast Cells

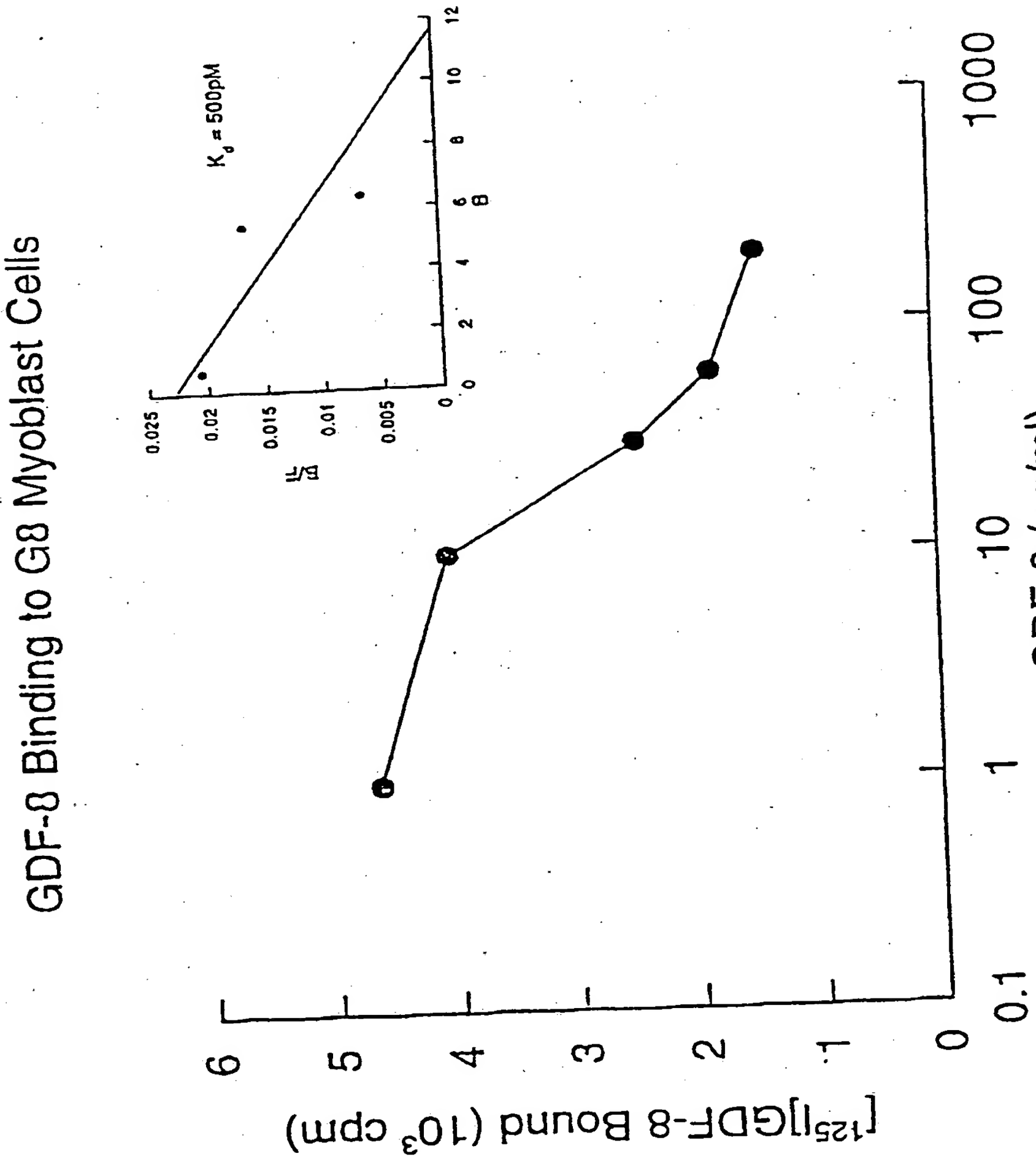
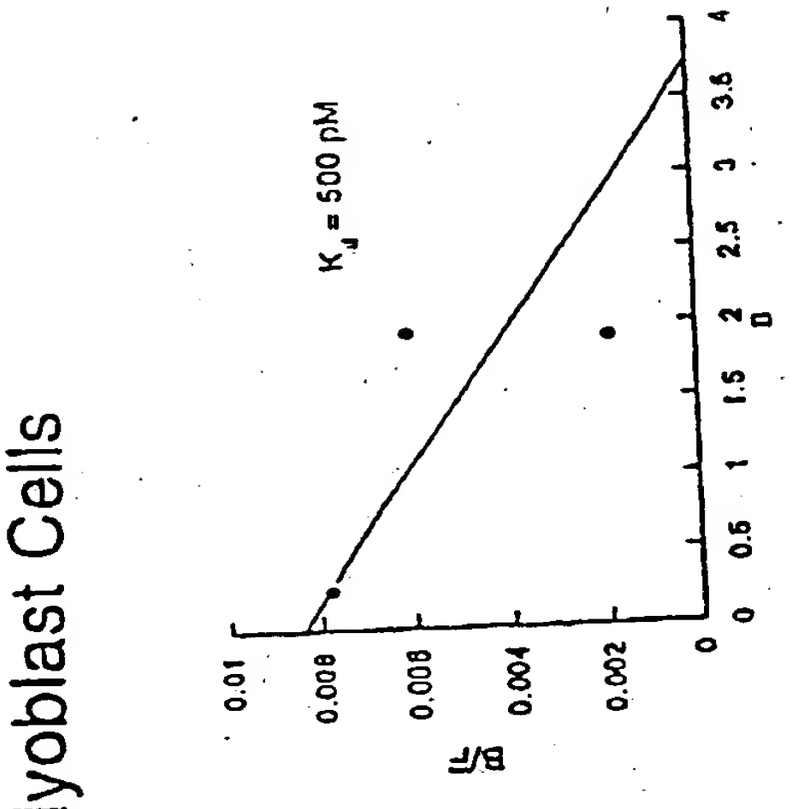


Figure 9

GDF-8 Binding to G7 Myoblast Cells



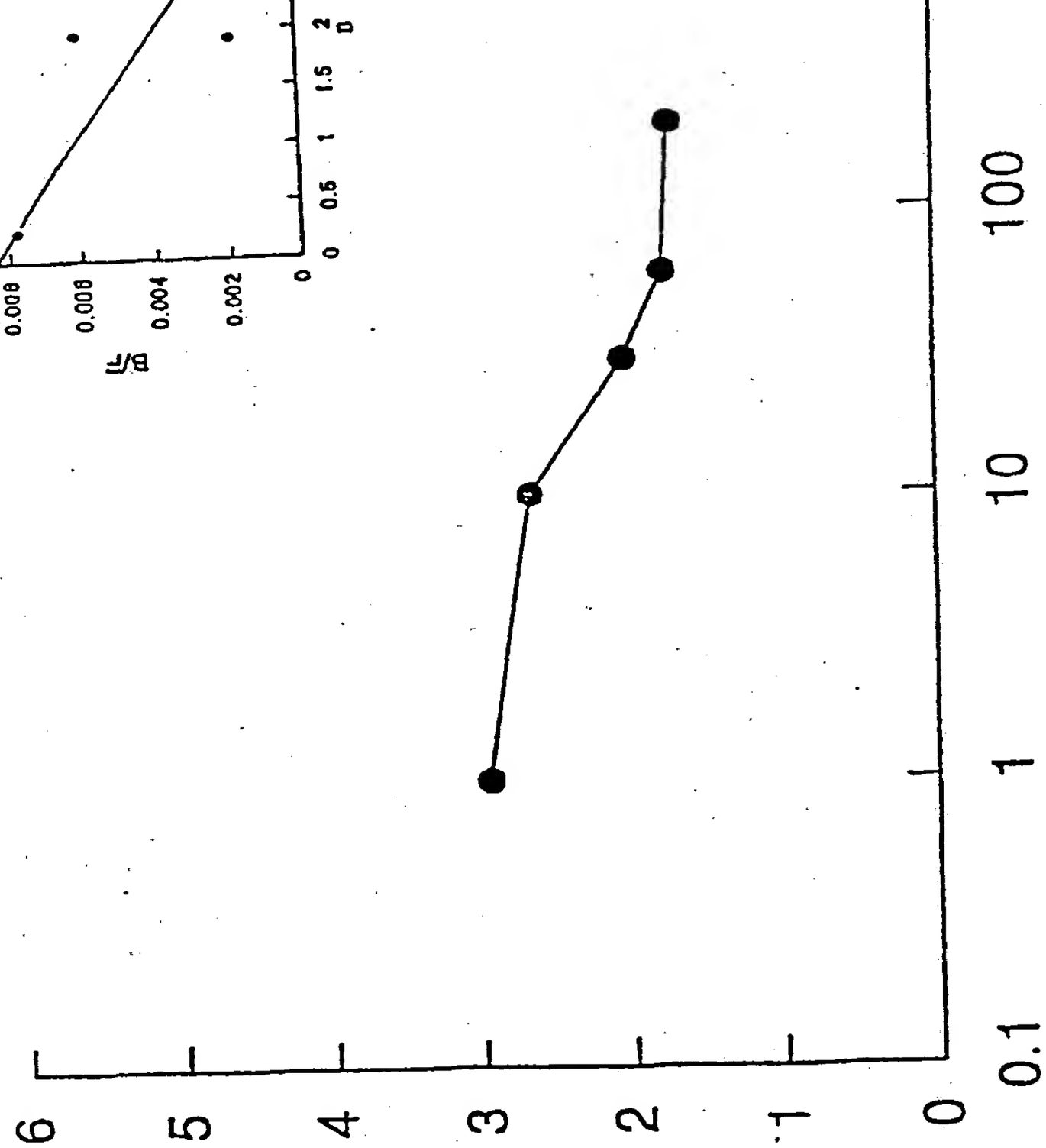
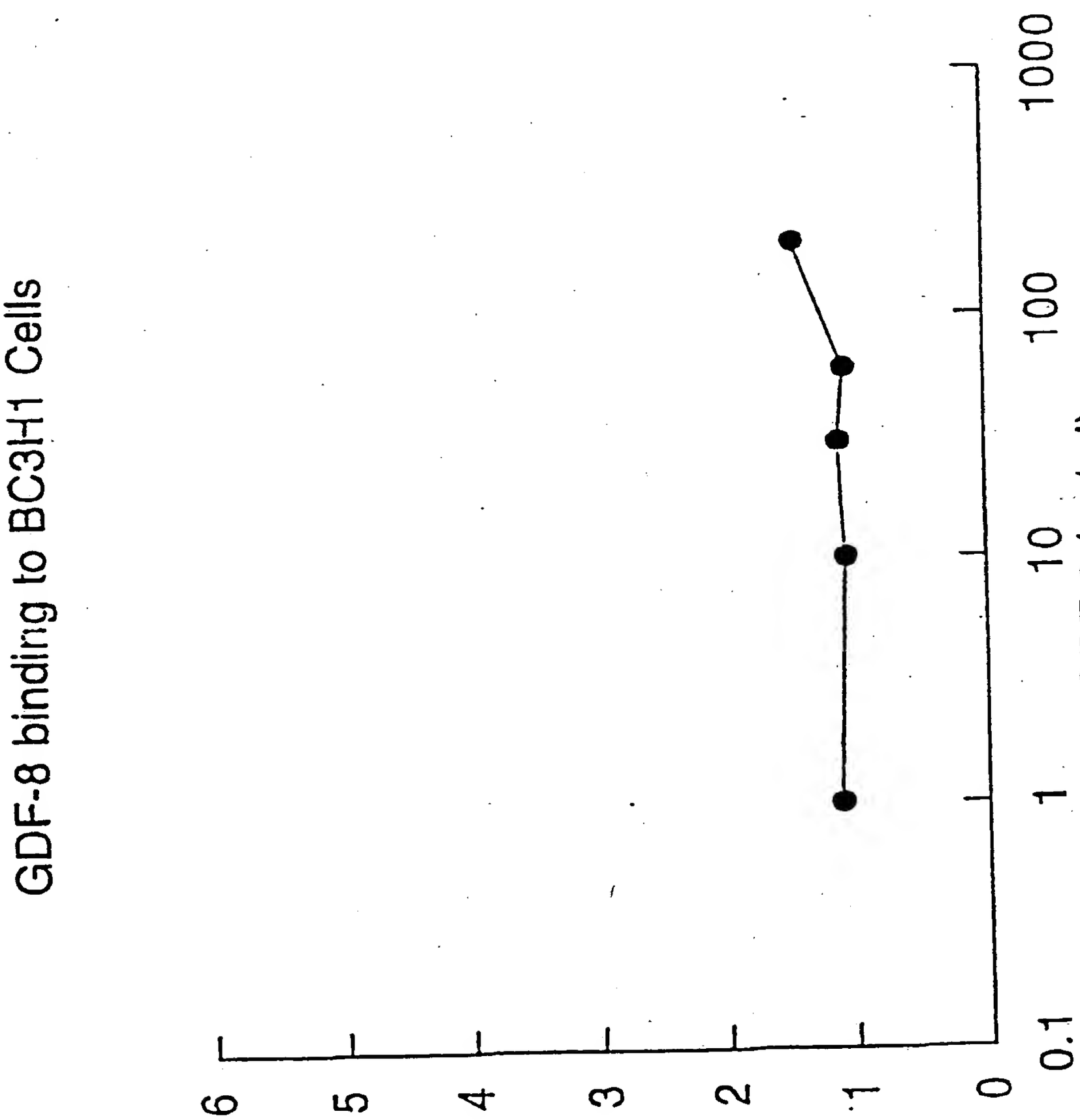


Figure 11


GDF-8 binding to BC3H1 Cells
[125I]GDF-8 Bound (10³ cpm)
0
1
2
3
4
5
6
0.1
1
10
100
GDF-8 (ng/ml)

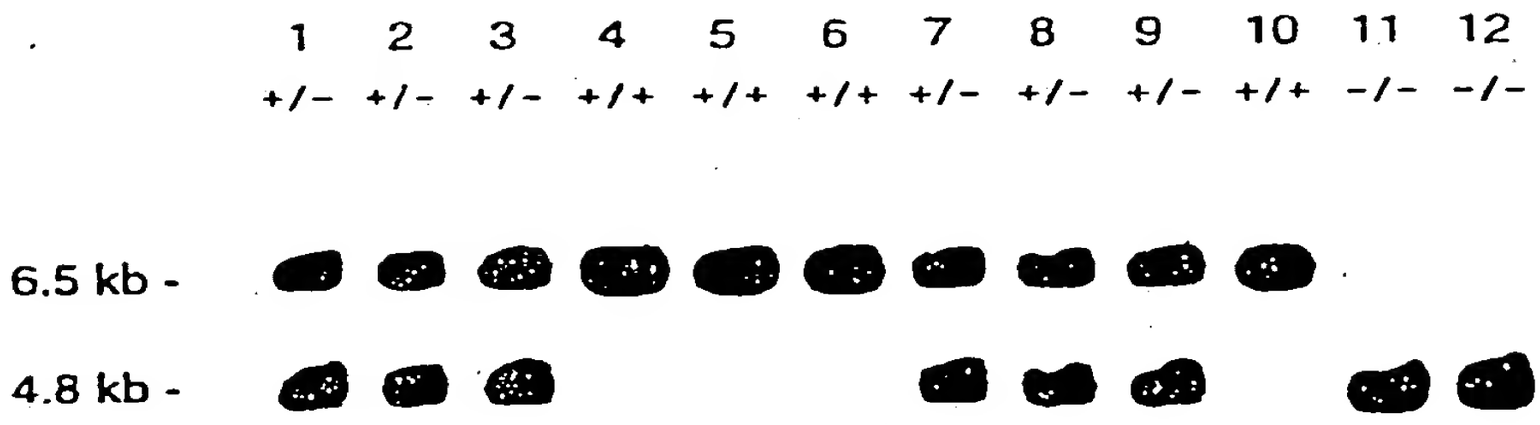


Fig. 12B

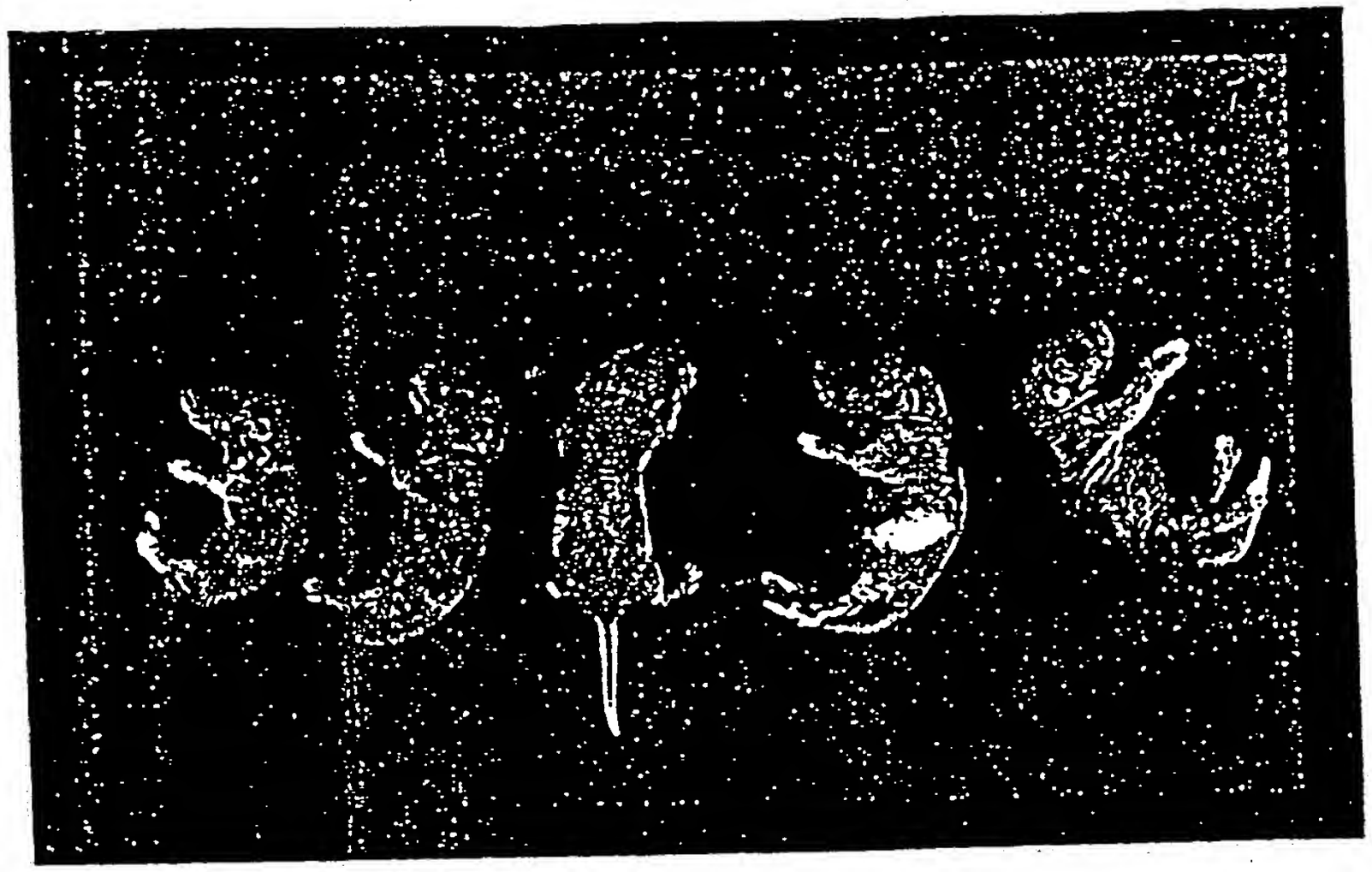


Fig. 14A

Fig.14E - 14G

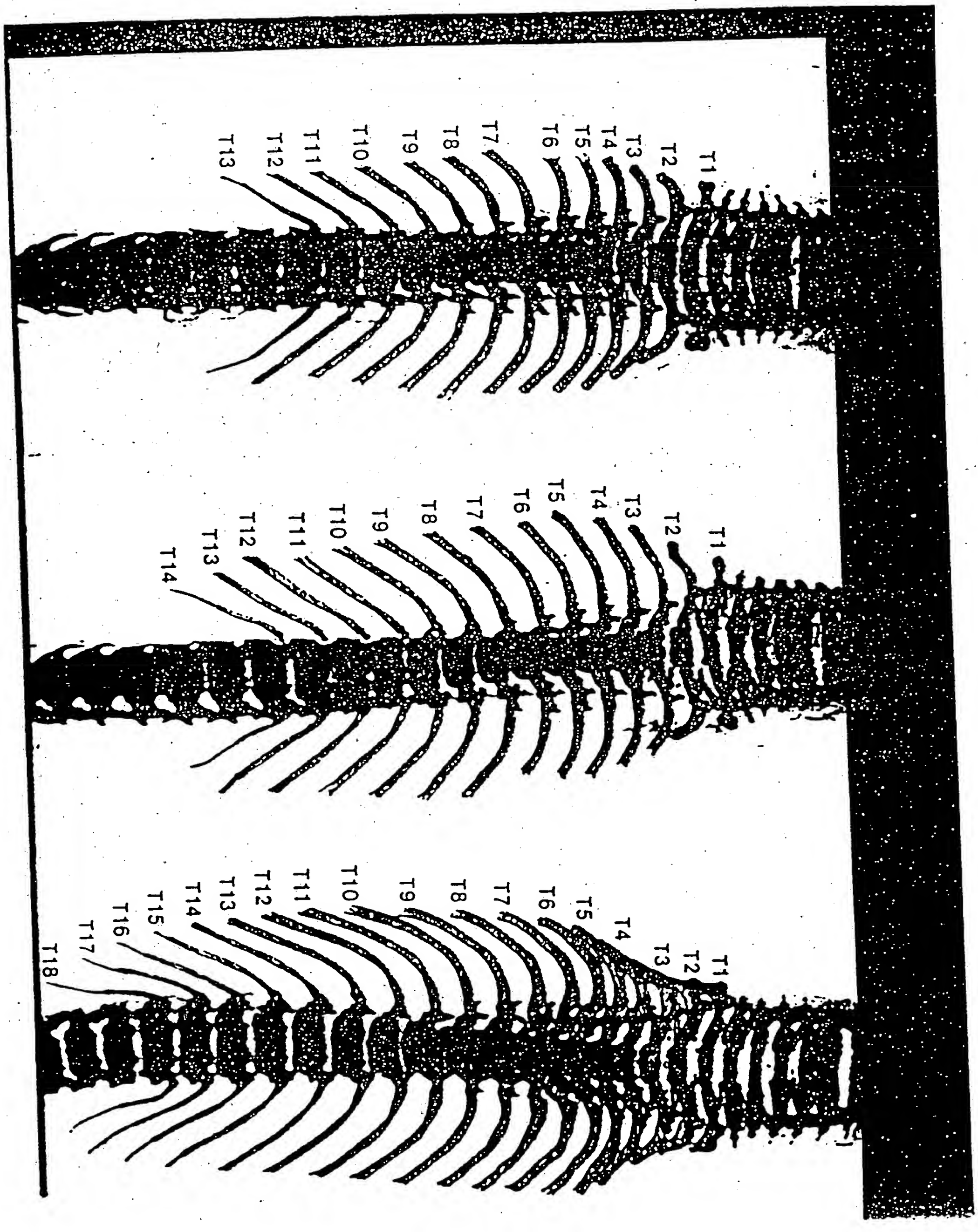


Fig. 15 Anterior transformations in wild-type, heterozygous and homozygous GDF-11 mice

	Wild-type		Heterozygous		Homozygous	
	Hybrid	129/SvJ	Hybrid	129/SvJ	Hybrid	129/SvJ
Presacral vertebrae[a]						
25	4	1	-	-	-	-
26	18	6	1	-	-	-
27	-	-	58	6	-	-
33	-	-	-	-	18	2
34	-	-	-	-	5	-
Vertebral pattern[a,b]						
C7 T13 L5	4	1	-	-	-	-
C7 T13 L6	18	6	-	-	-	-
C7 T13 L?[c]	-	1	-	-	-	-
C7 T14 L5	-	-	1	-	-	-
C7 T14 L6	-	-	58	6	-	-
C7 T17 L9	-	-	-	-	1	1
C7 T18 L8	-	-	-	-	17	1
C7 T18 L9	-	-	-	-	5	-
C7 T18 L?[c]	-	-	-	-	-	1
Anterior tuberculus on						
No vertebrae	-	1	-	-	-	-
C6	22	7	59	5	21	1
C6 and C7[d]	-	-	-	1	2	2
Attached/unattached ribs[b]						
7/6	22	8	-	-	-	-
8/6	-	-	59	6	-	-
10/7	-	-	-	-	-	1
10/8[e]	-	-	-	-	13	2
11/6	-	-	-	-	1	-
11/7	-	-	-	-	4	-
10 + 11/8 +7[f]	-	-	-	-	5	-
Longest spinous process on						
T2	22	5	41	-	2	-
T3	-	-	6	6	16	-
T2 + T3 equal	-	1	8	-	1	-
T3 + T4 equal	-	-	-	-	-	2
Transitional spinous process on						
T10	22	8	3	-	-	-
T11	-	-	56	6	-	-
T12	-	-	-	-	1	-
T13	-	-	-	-	22	3
Transitional articular process on[g]						
T10	22	8	1	-	-	-
T11	-	-	58	6	-	-
T13	-	-	-	-	23	3

[a]Vertebrae that were lumbar on one side and sacral on the other were scored as sacral. These vertebrae were seen in 2 wild-type, 3 heterozygous and 8 homozygous mutants in the hybrid background.
[b]One hybrid heterozygous, 9 hybrid homozygous and 2 129/SvJ homozygous mutants had rudimentary ribs on the most caudal thoracic segment.
[c]The number of lumbar vertebrae could not be counted due to extensive fusion of lumbar segments.
[d]These animals had a unilateral transformation of the anterior tuberculi. One 129/SvJ homozygous mutant retained one tuberculus on C6 but had bilateral tuberculi on C7.
[e]One 129/SvJ homozygous mutant had the first rib attached to the second rather than the sternum on one side only. Ten ribs were attached to the sternum on the other side.
[f]Ribs were asymmetrically attached.
[g]One wild-type 129/SvJ had one transitional articular process on T10 and one on T11 (scored as T10). One hybrid heterozygous mutant mice had one process on T11 and one on T12 (scored as T11).

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/15598

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 3-22 and 25-41
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Please See Extra Sheet.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.